Combining ALS-inhibiting Herbicides with the Fungal Pathogen *Mycoleptodiscus terrestris* for Control of Hydrilla

by Judy F. Shearer and Linda S. Nelson

PURPOSE: This technical note describes the results of multiple growth chamber studies conducted to evaluate the effectiveness of three ALS-inhibiting herbicides (penoxsulam, imazamox, and bispyribac-sodium) and a fungal pathogen applied alone and in combination with one another, as a potential method for controlling the nuisance submersed plant, hydrilla (*Hydrilla verticillata* (L.f.) Royle).

BACKGROUND: Numerous studies have shown that combining the indigenous fungal pathogen, *Mycoleptodiscus terrestris* (Gerd.) Ostazeski, hereafter referred to as Mt, with low doses of herbicides has excellent potential as an integrated strategy for long-term management of hydrilla and Eurasian watermilfoil (*Myriophyllum spicatum* L.) (Netherland and Shearer 1996; Nelson et al. 1998; Shearer and Nelson 2002; Nelson and Shearer 2005). With the advent of resistance to fluridone (1-methyl-3phenyl-5[3-(trifluoromethyl)phenyl]-4 (1*H*)-pyridinone) in hydrilla populations, it became essential to find new products for hydrilla management. Recently two new herbicides, penoxsulam (2-(2,2-difluoroethoxy)-*N*-(5,8-dimethoxy[1,2,4]triazolo[1,5-*c*]pyrimidin-2-yl)-6-(trifluoromethyl)benzenesulfonamide) and imazamox (2-[4,5-dihydro-4 methyl-4-(1-methylethyl)-5oxo-1*H*-imidazol-2-yl]-5-(methoxymethyl)-3-pyridinecarboxylic acid), have undergone registration and a third, bispyribac-sodium (sodium 2,6-bis[(4,6-dimethoxy-2-pyrimidinyl)oxy]benzoate) is currently being evaluated under an Experimental Use Permit (EUP) for aquatic use. The three herbicides are classified in different herbicide families but all have a similar mode of action; inhibition of the enzyme acetolactate synthase (ALS) (Koschnick et al. 2007). Current thought is that inhibition of ALS leads to starvation of a plant to the amino acids, isoleucine, valine, and leucine, and is the primary mechanism by which ALS herbicides cause plant death (Tranel and Wright 2002). Similar to fluridone, ALS inhibitors require extended contact times ($\geq$ 60 days) at designated use rates (ppb range) to achieve > 90 percent plant control. Reducing contact time requirements by integrating these herbicides with Mt would improve hydrilla control in areas where contact time is influenced by water exchange. Previous studies have shown that herbicide contact time can be reduced by as much as 50 percent when combining herbicides with Mt as a simultaneous treatment (Netherland and Shearer 1996; Nelson et al. 1998; Shearer and Nelson 2002).

Another important reason for examining the integration of ALS herbicides with Mt is to identify resistance management strategies for this new line of chemistry for aquatic systems. It has been reported that evolution of resistance to ALS-inhibiting herbicides has been rapid in terrestrial plant species (Ashigh and Tardif 2006; Beckie 2006). While it is unknown if and how quickly resistance will develop in aquatic plants, identifying alternate application strategies for this class of herbicides will ensure maintenance of viable tools for hydrilla control in the future. Integrating control strategies, such as herbicides + pathogens, can also minimize the selection pressure on weed populations, thereby reducing the risk for development of herbicide resistance (Buhler et al. 2000; Buhler 2002; Beckie 2006).

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Mycoleptodiscus terrestris is currently undergoing development as a mycoherbicide at the U.S. Department of Agriculture, Agriculture Research Service, National Center for Agricultural Utilization Research (USDA-ARS-NCAUR) Crop Protection Research Laboratory, Peoria, IL, in cooperation with the U.S. Army Engineer Research and Development Center (USAERDC), Vicksburg, MS (Shearer 2002; Shearer and Jackson 2003, 2006) and SePRO Corporation, Carmel, IN. The mycoherbicide currently under evaluation is a granular product composed primarily of aggregates of melanized hyphae or microsclerotia that are dried to a moisture content between 5 and 10 percent.

The objectives of the ALS herbicide/Mt studies were to: 1) evaluate the efficacy of combining ALS-inhibitor herbicides with Mt; 2) identify the effect of herbicide rate and contact time on treatment performance; and 3) assess the interaction between ALS herbicides and Mt.

MATERIALS AND METHODS: Studies were conducted in 55-L aquariums located in controlled-environment growth chambers at the USAERDC. Conditions in the growth chambers were maintained for optimal hydrilla growth: 22 $\pm$ 1 C with a light intensity of 580 $\pm$ 50 µmol m^{-2} sec^{-1} and a 14:10-hr light-dark photoperiod.

Due to the requirements of these studies and availability of good plant stock, hydrilla was obtained from different locations. For Study 1, hydrilla was collected from culture ponds at the U.S. Army Corps of Engineers Lewisville Aquatic Ecosystem Research Facility (LAERF), Lewisville, TX. Hydrilla used in Studies 2 and 3 was collected from the Rainbow River, FL.

Sediment was collected from Brown's Lake, Vicksburg, MS, and amended with ammonium chloride at a rate of 200 mg NH_4Cl L^{-1} sediment. Because the studies were long term (90 days) Osmocote (19-6-12) (Scotts-Sierra Horticultural Products Company, Marysville, OH) was placed in the bottom of plastic cups (946 ml) and overlain with sediment resulting in a use rate of 2.1 g Osmocote L^{-1} sediment. Four apical stem cuttings of hydrilla (approximately 15 cm in length) were planted into the sediment-filled cups. After planting, a thin layer of silica sand was added to the sediment surface to prevent sediment and nutrient dispersion into the water column. Four cups of plants were placed in each aquarium that had been pre-filled with 52 L of the aquatic plant culture solution developed by Smart and Barko (1984). Air was gently bubbled in each aquarium to provide circulation. Once weekly, one half the volume of culture solution was replaced in each aquarium to minimize nuisance algal growth. Plants were established under these conditions for approximately 28 days prior to treatment. At the time of treatment, plants had grown to the water surface of each aquarium.

Fungal inoculum of Mt (NRRL #30559) was prepared at the USDA-ARS-NCAUR using protocols as described in Shearer and Jackson (2006). Following 4 days incubation in a 100-L fermentor (B. Braun Biostat D, Sartorius, Melsungen, Germany), 20-L aliquots of whole culture were diluted with 80 L of sterile water and poured into a metal vat connected to a rotary drum vacuum filter that had been coated with 4 kg of Hyflo Supercel (Celite Corporation, Santa Barbara, CA). The mixture was pumped onto the Hyflo filter cake and as the drum rotated, the Mt/Hyflo cake was cut into layers that were passed successively through a Quadro grinder (Quadro Engineering, Waterloo, Ontario, Canada) to achieve final granule sizes $\leq$ 1397 µm. The granules were air dried overnight to a moisture content between 5 and 10 percent, placed in polyethylene bags, vacuum sealed, and refrigerated at 4 °C until needed.

At inoculation time the vacuum-sealed bags were opened and the inoculum weighed into plastic weigh boats and sprinkled over the water surface of the aquariums. As the inoculum rehydrated, the granules began to slowly fall through the water column and lodged on leaf blades or in leaf axils. The bags of inoculum were resealed with a vacuum packer and returned to cold storage after each use.

Study 1 - Penoxsulam + Mt. A concentrated stock solution of penoxsulam was prepared by dissolving an aqueous formulation (as Galleon™ SC (240 g ai L^{-1}), SePRO Corporation, Carmel, IN) into glass-distilled water. The stock solution was mixed using a stir plate and magnetic stir bar and was prepared approximately 0.5 hr prior to treatment. Both the fungal inoculum and the herbicide stock solution were dispensed to the water surface in each aquarium. Treatments included 10 µg L^{-1} penoxsulam, Mt alone at 0.02 g L^{-1}, and 0.01 g L^{-1} Mt, combined treatments of herbicide + pathogen, and untreated controls. Combined treatments were applied simultaneously. Herbicide contact times were 21, 35, 56, and 90 days. Following each designated contact time, aquariums were emptied and refilled with fresh water three times to remove treatment residues (namely herbicide residues). For aquariums receiving a 90-day contact to penoxsulam, culture solution was periodically added to replenish that which had evaporated, thus maintaining herbicide concentrations. Shoot biomass was harvested over time at 28, 42, 70, and 90 days after treatment (DAT), dried to a constant weight, and weights were recorded.

Study 2 - Imazamox + Mt. A concentrated stock solution of imazamox was prepared by dissolving an aqueous formulation (as Clearcast™ (120 g ae L^{-1}), BASF Corporation, Research Triangle Park, NC) into glass-distilled water. The stock solution was mixed using a stir plate and magnetic stir bar and was prepared approximately 0.5 hr prior to treatment. Both the fungal inoculum and the herbicide stock solution were dispensed to the water surface in each aquarium. Treatments included 10, 25, and 50 µg L^{-1} imazamox, 0.03 g L^{-1}, and 0.015 g L^{-1} Mt, combined treatments of herbicide + pathogen, and untreated controls. Combined treatments were applied simultaneously. Shoot biomass was harvested over time at 28, 42, 70, and 90 DAT, dried to a constant weight, and weights were recorded.

Study 3 - Bispyribac-sodium + Mt. A concentrated stock solution of bispyribac-sodium was prepared by dissolving a wettable powder formulation of bispyribac-sodium (as Velocity™ (80 percent a.i), Valent U.S.A. Corporation, Walnut Creek, CA) into glass-distilled water. Both the fungal inoculum and the herbicide stock solution were dispensed to the water surface in each aquarium. Treatments included 5, 10, and 20 µg L^{-1} bispyribac-sodium, 0.03 g L^{-1}, and 0.015 g L^{-1} Mt, combined treatments of herbicide + pathogen, and untreated controls. Combined treatments were applied simultaneously. Shoot biomass was harvested over time at 28, 42, 70, and 90 DAT, dried to a constant weight, and weights were recorded.

For all studies, treatments were randomly assigned to aquariums and replicated three times. Data were subjected to analysis of variance procedures using Statistica 7.1 (StatSoft, Tulsa, OK). Where appropriate, the data were transformed using log(x+1) to meet the assumptions for normality and equality of variance. When significant treatment effects were found, means were separated using Fisher's protected LSD test at the 0.05 level of significance. For simplicity and clarity of presentation, non-transformed data are presented with statistical interpretation based upon transformed data.

RESULTS AND DISCUSSION: The Mt fungal inoculum that was used in all the experiments came from the same batch that was harvested on July 10, 2007, and dried overnight. By the time it was used for experimentation it had been in storage for 5 months for Study 1 and 7 months for Studies 2 and 3. To date, long-term storage stability of the dried material has not been fully evaluated. Some preliminary findings had indicated that dried material would remain viable for at least 6 months in cold storage with a slight loss of efficacy on hydrilla.[1] For these reasons the dose rate was increased for Studies 2 and 3 by 0.015 g L^{-1}, and 0.03 g L^{-1}, respectively for low and high dose treatments.

Study 1 – Penoxsulam + Mt. Treatment effects on hydrilla shoot biomass are presented in Table 1. Between the 28-day harvest and the 90-day harvest, untreated plant biomass increased almost fourfold. Application of Mt at 0.01 g L^{-1} significantly reduced hydrilla biomass compared to the untreated control by 28 DAT, however the treated plants recovered quickly and by final harvest the biomass had increased almost twentyfold and was not significantly different than the untreated control. Because the Mt application rates used in the experiment were sub-lethal, it was expected that plants that received the higher Mt rate would also recover. This appeared to be the trend as biomass weights recorded at 42 and 70 DAT indicated plants were beginning to recover but at the final harvest the mean biomass recorded was almost sevenfold less than at 70 DAT. Three possibilities may have accounted for the low biomass recorded at final harvest. First, there could have been variation in the amount of biomass between pots although at the time the experiment was started, every effort was made to assure that all pots had equal amounts of plant material. Second, when Mt was applied it was allowed to naturally dissipate through the water column. Plants in some pots may have received more inoculum and thus had more disease than plants in other pots. In these cases, recovery rates may have been delayed. Thirdly, Mt can produce secondary inoculum in the form of spores. The exact timing of this event could incite a new round of infection, resulting in disease and with it a decrease in plant biomass.

When applied alone, the removal of penoxsulam at 21, 35, and 56 days resulted in plant regrowth and an increase in shoot biomass by 90 DAT. Although shoot biomass decreased over the 90-day exposure to 10 µg L^{-1} penoxsulam, biomass was reduced only 78.2 percent compared to the untreated control. In terms of management, a treatment strategy that results in less than 80-percent control of plant biomass can be considered an unsuccessful operational treatment. However, in field situations these same rates have resulted in 100-percent control of hydrilla.[2] It is thought that additional stress factors that are encountered in the field additionally contribute to the effectiveness of penoxsulam. The only combined treatments that resulted in less than 80-percent control 90 DAT were low rates of Mt with penoxsulam exposure times of 21 and 35 days. The combination of Mt applied at 0.02 g L^{-1} with 10 µg L^{-1} penoxsulam for a 21-day exposure time might also be a questionable combination for management purposes as biomass was reduced only 84.7 percent compared to the untreated control. However all other combined treatments reduced biomass greater than 90 percent. By combining the higher rate of Mt with penoxsulam, exposure time could be reduced by 55 days (from 90 to 35 days). At longer exposure times Mt rates could be reduced by half (from 0.02 g L^{-1} to 0.01 g L^{-1}) without compromising efficacy.

[1] Personal Observation. 2008. Dr. Judy Shearer, Environmental Laboratory, U.S. Army Engineer Research and Development Center.
[2] Personal Communication. 2008. Mark Heilman, SePRO Corporation, Carmel, IN.

Table 1
Effects of Penoxsulam, *M. terrestris*, and Combinations of Penoxsulam and *M. terrestris* on Shoot Biomass of Hydrilla (Rates and contact time (days) varied among treatments.)

Treatment	Mean Dry Weight Biomass (g)			
	28 DAT	42 DAT	70 DAT	90 DAT
Untreated Control	3.45 a	4.02 ab	7.55 a	13.45 a
M. terrestris alone				
0.01 g L^{-1}	0.38 bcd	3.02 ab	7.04 a	7.52 abc
0.02 g L^{-1}	0.04 d	1.16 cd	3.38 bcd	0.48 fg
Penoxsulam alone (contact time)				
10 µg L^{-1} (21 days)	3.09 a	3.94 ab	3.64 abc	10.43 ab
10 µg L^{-1} (35 days)	3.01 a	4.01 ab	1.87 bcde	5.34 bc
10 µg L^{-1} (56 days)	3.13 a	3.09 ab	1.39 def	3.50 cd
10 µg L^{-1} (90 days)	3.47 a	4.66 a	2.27 bcd	2.93 cde
Penoxsulam + *M. terrestris* (contact time)				
10 µg L^{-1} + 0.01 g L^{-1} (21 days)	0.49 bc	1.91 bc	4.21 ab	9.46 ab
10 µg L^{-1} + 0.02 g L^{-1} (21 days)	0.06 d	0.39 d	1.67 def	2.06 def
10 µg L^{-1} + 0.01 g L^{-1} (35 days)	0.57 b	1.06 cd	0.80 defg	4.36 cd
10 µg L^{-1} + 0.02 g L^{-1} (35 days)	0.20 bcd	0.30 d	0.50 fg	1.01 fg
10 µg L^{-1} + 0.01 g L^{-1} (56 days)	0.59 b	0.96 cd	0.27f g	1.07 efg
10 µg L^{-1} + 0.02 g L^{-1} (56 days)	0.15 cd	0.24 d	0.04 g	0.06 g
10 µg L^{-1} + 0.01 g L^{-1} (90 days)	0.47 bc	0.58 d	0.44 fg	0.30 fg
10 µg L^{-1} + 0.02 g L^{-1} (90 days)	0.14 cd	0.51 d	0.06 g	0.16 g

Note: Within each column, values followed by a different letter are significantly different according to Fisher's LSD at $p \leq 0.05$; n = 3; DAT = days after treatment.

Study 2 – Imazamox + Mt. Treatment effects on hydrilla shoot biomass are presented in Table 2. Between the 28-day harvest and the 90-day harvest, untreated plant biomass increased 2.3-fold. Compared to the untreated control, imazamox applied alone at the lowest rate did not significantly reduce shoot biomass over time. It appeared that hydrilla began to regrow by 42 DAT and continued to recover over time. At the 28-day harvest, shoot biomass of plants treated with medium and high imazamox rates were not significantly different than the untreated control; however, at all subsequent harvest dates, exposure to medium and high imazamox rates significantly reduced shoot biomass compared to the untreated control. At the time of the final harvest, imazamox applied at the medium rate provided only 58-percent control, which would not be considered a successful operational treatment. While percent control reached 86 percent using the highest imazamox rate, it did not provide better control than either of the Mt treatments applied alone or in combination with imazamox.

It was thought that additional time in storage would compromise the viability of Mt dried material; therefore, application rates were increased slightly over what was used for the penoxsulam study. The intent was to use sub-lethal doses for all Mt applications to try to detect any interactions with various rates of imazamox that would provide better control than either product used alone.

Table 2
Effects of Imazamox, *M. terrestris*, and Combinations of Imazamox and *M. terrestris* on Shoot Biomass of Hydrilla

Treatment	Mean Dry Weight Biomass (g)			
	28 DAT	42 DAT	70 DAT	90 DAT
Untreated Control	3.21 ab	5.13 a	7.87 a	7.51 a
M. terrestris alone				
0.015 g L^{-1}	0.01 c	0.00 e	0.01 d	0.61 cd
0.030 g L^{-1}	0.04 c	0.05 de	0.22 d	0.67 cd
Imazamox alone				
10 µg L^{-1}	2.95 b	4.20 ab	6.42 a	6.75 ab
25 µg L^{-1}	4.37 a	2.37 c	3.06 b	3.15 b
50 µg L^{-1}	3.44 ab	3.03 bc	1.24 c	1.07 c
Imazamox + *M. terrestris*				
10 µg L^{-1} + 0.015 g L^{-1}	0.34 c	0.52 d	0.18 d	0.41 cd
10 µg L^{-1} + 0.030 g L^{-1}	0.01 c	0.02 e	0.00 d	0.01 d
25 µg L^{-1} + 0.015 g L^{-1}	0.02 c	0.01 e	0.02 d	0.00 d
25 µg L^{-1} + 0.030 g L^{-1}	0.07 c	0.01 e	0.00 d	0.01 d
50 µg L^{-1} + 0.015 g L^{-1}	0.35 c	0.22 de	0.15 d	0.07 cd
50 µg L^{-1} + 0.030 g L^{-1}	0.14 c	0.04 de	0.02 d	0.01 d

Note: Within each column, values followed by a different letter are significantly different according to Fisher's LSD at $p \leq 0.05$; n = 3; DAT = days after treatment.

Unfortunately, the Mt rates used alone proved to be lethal to hydrilla in this study. Applied at both low and high rates, hydrilla shoot biomass was significantly less than the untreated controls at all harvest dates and although biomass increased slightly over time by the final harvest, percent control was 92 and 91 percent for low and high dose treatments, respectively. Very slight increases in percent control were achieved with the addition of imazamox at all rates, but none were significantly different than Mt used alone.

Study 3 – Bispyribac-sodium + Mt. Treatment effects on hydrilla shoot biomass are presented in Table 3. Between the 28-day harvest and the 90-day harvest, untreated plant biomass increased almost fourfold. At 28 DAT only bispyribac-sodium applied at the highest rate significantly reduced hydrilla shoot biomass compared to the untreated control. By 42 DAT, bispyribac-sodium treated plants showed an increase in shoot biomass at all application rates and no significant biomass differences were detected between the treated plants and the untreated control. By 90 DAT all bispyribac-sodium treated plant biomass was significantly different than the untreated controls, but none of the rates provided acceptable management control levels. For low, medium, and high bispyribac-sodium treatment rates, amount of hydrilla control was 56, 71, and 73 percent, respectively, much less than Mt applied alone where low and high treatment rates provided 100- and 95-percent control, respectively.

As mentioned above for Study 2, the Mt rates that were used in the study were lethal to hydrilla thus overshadowing any effects that combination treatments might have provided. At each harvest date there were no significant differences between Mt applied alone and in combination with bispyribac-sodium.

Table 3
Effects of Bispyribac-sodium, *M. terrestris*, and Combinations of Bispyribac-sodium and *M. terrestris* on Shoot Biomass of Hydrilla

Treatment	Mean Dry Weight Biomass (g)			
	28 DAT	42 DAT	70 DAT	90 DAT
Untreated Control	3.21 ab	5.13 a	7.87 a	7.51 a
M. terrestris alone				
0.015 g L^{-1}	0.15 d	0.25 b	0.00 d	0.03 c
0.030 g L^{-1}	0.20 d	0.39 b	0.24 d	0.52 c
Bispyribac-sodium alone				
5 µg L^{-1}	3.83 a	5.37 a	3.57 ab	4.98 b
10 µg L^{-1}	2.25 b	5.76 a	3.27 b	3.24 b
20 µg L^{-1}	1.41 c	4.24 a	1.03 c	3.02 b
Bispyribac-sodium + *M. terrestris*				
5 µg L-1 + 0.015 g L-1	0.02 d	0.02 b	0.01 d	0.00 c
5 µg L-1 + 0.030 g L-1	0.01 d	0.03 b	0.00 d	0.00 c
10 µg L-1 + 0.015 g L-1	0.28 d	0.03 b	0.02 d	0.00 c
10 µg L-1 + 0.030 g L-1	0.02 d	0.01 b	0.00 d	0.00 c
20 µg L-1 + 0.015 g L-1	0.02 d	0.08 b	0.02 d	0.00 c
20 µg L-1 + 0.030 g L-1	0.01 d	0.01 b	0.00 d	0.00 c

Note: Within each column, values followed by a different letter are significantly different according to Fisher's LSD at $p \leq 0.05$; n = 3; DAT = days after treatment.

Studies are ongoing at the present time to determine the shelf life of the dried Mt product. Preliminary results from these studies indicate that the product can easily survive 7 months in cold storage without compromising fungal virulence. In natural populations microsclerotia are produced in response to adverse environmental conditions. In fermentation, microsclerotia that develop in the broth medium as nutrients, particularly carbon and nitrogen, become limiting (Shearer and Jackson 2006). Germination of the microsclerotia occurs when conditions are favorable for growth and a host is available. Removal from cold storage and application of the product into warm water containing hydrilla in all likelihood serves to break dormancy of the propagules inducing them to germinate hyphally and sporogenically, resulting in rapid disease development and subsequent hydrilla decline.

The results of Study 1 demonstrated that integrating Mt with penoxsulam has the potential to provide better and longer term control of hydrilla than either herbicide or fungal pathogen alone. Herbicide use rates and contact time were significantly reduced when combining these two agents. Similar to other studies that evaluated Mt combined with herbicides (Netherland and Shearer 1996; Nelson et al. 1998; Shearer and Nelson 2002), Study 1 data support the potential of integrated weed management as an effective, reduced-risk alternative for control of hydrilla. As the three herbicides used in the studies are all ALS inhibitors and have similar modes of action, it would be expected that if Mt dose rates had been reduced to sub-lethal levels, the imazamox and bispyribac-sodium studies would also have documented the advantage of combining low rates of Mt with the herbicides for hydrilla management.

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POINTS OF CONTACT: For additional information, contact the authors, Dr. Judy F. Shearer (601-634-2516, Judy.F.Shearer@usace.army.mil), and Dr. Linda S. Nelson (601-634-2656, Linda.S.Nelson@usace.army.mil), or the manager of the Aquatic Plant Control Research Program, Robert C. Gunkel (601-645-3722, Robert.C.Gunkel@usace.army.mil). This technical note should be cited as follows:

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